Salicylic acid mediates the reduced growth of lignin down-regulated plants

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Down-regulation of the enzyme hydroxycinnamoyl CoA: shikimate hydroxycinnamoyl transferase (HCT) in thale cress (Arabidopsis thaliana) and alfalfa (Medicago sativa) leads to strongly reduced lignin levels, reduced recalcitrance of cell walls to sugar release, but severe stunting of the plants. Levels of the stress hormone salicylic acid (SA) are inversely proportional to lignin levels and growth in a series of transgenic alfalfa plants in which lignin biosynthesis has been perturbed at different biosynthetic steps. Reduction of SA levels by genetically blocking its formation or causing its removal restores growth in HCT–down-regulated Arabidopsis, although the plants maintain reduced lignin levels. SA-mediated growth inhibition may occur via interference with gibberellic acid signaling or responsiveness. Our data place SA as a central component in growth signaling pathways that either genetically impact growth.

Reduced cells with highly reduced cell-wall recalcitrance without negatively impacting growth.

biofuel crops | defense signaling | lignin modification | Medicago truncatula

The phenylpropanoid-derived polymer lignin cross-links plant secondary cell walls to provide mechanical strength and hydrophobicity to the vascular system as well as contributes to defense against biotic stress (1). Reducing lignin levels by genetic manipulation improves both forage digestibility and processing of lignocellulosic biomass for liquid biofuel production (2). However, transgenic plants down-regulated in the hydroxycinnamoyl CoA: shikimate hydroxycinnamoyl transferase (HCT) enzyme, which have a strong reduction in lignin levels, show severe defects in growth (3–5). Similar, although less severe, effects are observed in plants down-regulated in some, but not all, of the other enzymes of the monolignol pathway (5–10).

The dwarf phenotype of HCT–down-regulated Arabidopsis plants can be alleviated by restoring lignin accumulation through expression of a Selaginella enzyme that bypasses the reactions catalyzed by HCT (4), suggesting that structural alterations to the partially lignified secondary cell walls are in some way linked to the growth defects. Such structural alterations include increased extractability of pectic polysaccharides (11). It is not clear whether it is possible to strongly reduce lignin content in plant cell walls without causing structural changes that will lead to deleterious effects.

HCT–down-regulated alfalfa plants contain increased levels of the stress hormone salicylic acid (SA) and SA-inducible pathogenesis-related (PR) protein transcripts (11). PR gene expression can be induced by cell-wall pectic fragments via processes involving SA (12, 13), and SA can impact plant growth and development through largely unknown mechanisms (14, 15). A previous report that lignin reduction in HCT–down-regulated plants results from flavonoid-mediated inhibition of auxin transport has recently been refuted (4).

Levels of SA correlate with the extent of lignin reduction in a series of transgenic alfalfa lines expressing antisense or RNAi constructs independently targeting seven enzymes of the monolignol pathway (16). The potential relationship between SA levels and lignin is intriguing because SA is biosynthetically related to lignin (17). Two major routes have been proposed for SA biosynthesis in plants. The initial pathway via isochorismate, derived from the shikimic acid pathway, is supported genetically (18), but enzymes that convert isochorismate to SA have yet to be shown in plants. Similarly, the previously favored pathway via cinnamate is believed to involve a benzozate 2-hydroxylase (19), but no gene encoding this enzyme has been shown in plants. Theoretically, back-up of flux into the lignin pathway could result in increased production of SA via either route. Alternatively, SA accumulation might be a result of activation of endogenous defense responses by elicitor-active polysaccharides released from improperly lignified cell walls (11).

We show here that removal of SA by genetic approaches relieves growth inhibition but maintains low lignin levels in HCT–down-regulated Arabidopsis thaliana. Thus, there is no a priori reason why plants cannot be engineered to contain highly processable cell walls yet also produce abundant biomass. The effects of SA are associated with alterations in gibberellin (GA) responsiveness and signaling.

Results

Correlations Between SA Levels and Growth in a Population of Lignin-Modified Plants. To investigate whether SA levels were directly related to growth phenotypes, we examined a series of transgenic alfalfa lines in which lignin biosynthesis had been perturbed via antisense or RNAi-mediated down-regulation (2). The collection of plants analyzed consisted of three WT controls and sets of three independent transgenic events down-regulated independently at each of six successive steps of the monolignol pathway (24 plants grown in parallel) (Fig. 1). Lignin levels (thioacidolysis yields) in the first six internodes were from WT levels (130 mmol·g−1 dry weight) to as low as 30 mmol·g−1 dry weight and inversely correlated with SA levels (R2 = 0.796) (16). Stem height was inversely correlated with endogenous SA levels across this population of plants (Fig. L4). In addition, a positive correlation was observed between SA levels and the amount of cell-wall pectic material that was extractable by cold water from alcohol-insoluble cell-wall residues prepared from the aerial portions of the plants (Fig. 1B). Altogether, these data indicate that the previous observations of elevated SA levels and water-soluble pectic elicitors of PR protein induction in HCT–down-regulated alfalfa (11) are not specific to HCT down-regulation but reflect the extent of lignin reduction independent of the lignin pathway enzyme targeted.

Blocking SA Production Restores Growth to HCT–Down-Regulated A. thaliana Plants. We used A. thaliana to genetically separate reduced lignin from elevated SA levels because mutants of...
Arabidopsis in which SA biosynthesis is blocked are available. As in alfalfa (11), Arabidopsis HCT-RNAi lines are severely dwarfed compared with WT plants (Fig. 2A) (3) and exhibit increased SA levels and expression of the PR-1, PR-2, and PR-5 defense genes (Fig. S1A and B). SA and PR transcript levels are also elevated in the cinnamoyl CoA reductase 1 (ccr1) mutant of Arabidopsis (Fig. S2), suggesting that, as in alfalfa, elevated SA production is not restricted to plants with lignin modification targeted only to HCT.

SA biosynthesis may occur through either the isochorismate synthase (ICS) route or via benzoic acid derived from cinnamate (17). The fast-neutron mutagenesis ccr1 contains an exon deletion in the gene encoding ICS (20) that is implicated in SA biosynthesis (18). Homozygous sid2-2 plants have growth, basal SA levels, and HCT and PR transcript levels similar to those in WT plants (Fig. 2A–D). To determine the impact of blocking SA biosynthesis through loss of function of ICS on the growth of HCT–down-regulated plants, homozygous sid2-2 mutant plants were crossed with a WT SID2-2 allele. A control cross between WT and HCT-RNAi plants was performed in parallel. In the F1 population, heterozygous HCT-RNAi × WT plants exhibited reduced plant height similar to that in the HCT-RNAi dwarf parents, whereas heterozygous sid2-2 × HCT-RNAi progeny showed a growth phenotype intermediate between that of the parents. The heterozygous sid2-2 × hemizygous HCT-RNAi plants from the F1 population were crossed again with the sid2-2 null mutant. Among a population of 40 progeny, four genotypes were found at approximately equal frequency: sid2-2/SID2-2, sid2-2/sid2-2, sid2-2/SID2-2 with HCT-RNAi, and sid2-2/sid2-2 with HCT-RNAi. The HCT-RNAi × WT progeny retained their dwarf growth habit (Fig. 2A and B), high SA levels (Fig. 2C), and elevated PR transcript levels (Fig. 2D). In contrast, SA and PR transcript levels were reduced and growth was considerably restored in HCT-RNAi lines either homozygous or heterozygous for the sid2 mutation (Fig. 2A–D). The SA levels of the sid2-2 heterozygotes were intermediate, between those of the HCT-RNAi and sid2-2 or WT values (Fig. 2C). Heterozygous sid2 mutants in the WT background do not exhibit reduced SA levels after fungal infection (21); the apparent lack of haploinsufficiency with regard to the SA phenotype in the present crosses may be the result of the different HCT expression levels in infected plants in a WT background (such as in ref. 19) and the present uninfected plants with an HCT-RNAi background.

Extractable HCT enzymatic activity, staining of syringyl lignin with Maüle reagent (1), and lignin monomer thioacidolysis yields were strongly reduced in all lines expressing the HCT-RNAi construct, including those homozygous for the sid2 mutant with restored growth (Fig. 2E–G). The lignin composition of the HCT-RNAi lines in the sid2-2/sid2-2 background was typical of plants with reduced HCT expression, namely elevated hydroxyphenyl lignin units, and strongly reduced levels of guaiacyl and syringyl units (22). Thus, HCT–down-regulated Arabidopsis plants in which SA biosynthesis is blocked no longer display a dwarf phenotype, despite their strongly reduced lignin levels and drastically altered lignin composition.

Conversion of SA to Catechol Also Restores Growth to HCT–Down-Regulated A. thaliana Plants. Because of the potential of multiple pathways for SA biosynthesis, we sought additional genetic confirmation of the role of SA in the growth phenotype of HCT-RNAi plants. The HCT-RNAi line was therefore crossed with plants expressing the bacterial NahG gene, which encodes a salicylate hydroxylase that removes SA by converting it to catechol (23). NahG Arabidopsis plants have WT growth habit (Fig. 3A and D), and introduction of the NahG transgene into HCT-RNAi plants restored growth to WT levels and reduced SA and PR transcript levels (Fig. 3A–D) in a manner similar to the introduction of the sid2 mutation. The extractable HCT activity of the individual NahG:HCT-RNAi progeny plants analyzed varied considerably, as did the overall lignin level, both of which, however, were always lower than those of the NahG parent line (Fig. S3A and B). In addition, all NahG:HCT-RNAi lines showed the hydroxyphenyl lignin signature characteristic of HCT down-regulation (Fig. S3B). The elevation of HCT expression in many of the lines from this cross is likely the result of partial silencing of the 35S promoter derived HCT-RNAi by the additional copy of the 35S promoter driving the NahG gene. Importantly, however, HCT expression and lignin levels in some progeny lines were as low as in the HCT-RNAi parent line, but growth was normal (Fig. S3 B and C).

Reduction of SA-Mediated Defense Signaling Does Not Restore Growth to Low-Lignin Arabidopsis Plants. To test whether PR expression, distinct from SA production, was causing the growth defects in HCT-RNAi plants, we crossed HCT-RNAi plants with plants harboring a mutation in NPR1, a gene involved in signal transduction between SA and PR expression (24). HCT-RNAi lines homozygous for the npr1 mutation retained elevated SA levels (Fig. 3B) and exhibited a partial reduction (~50%) of PR5 and PR2 transcript levels, although PRI transcripts were unaltered (Fig. 3C). The growth of these plants remained severely stunted (Fig. 3A and D). These results are consistent with the fact that PR genes can be regulated by an alternative NPR1-independent pathway (25, 26); for example, constitutive PR gene expression in the cpr6-1 dominant mutation in Arabidopsis requires elevated SA levels but not NPR1 function (25).

Restoring Growth by Removal of SA Is Not Mediated by Altered Flavonoid Levels. Arabidopsis HCT-RNAi plants accumulate high levels of flavonoids because of metabolic spillover from the lignin to the flavonoid pathway, which shares 4-coumaroyl CoA, the
substrate for HCT, as a common early intermediate (3, 4). Flavonoid levels were reduced somewhat in the sid2-2 × HCT-RNAi plants, but they still contained significantly higher levels of flavonoids than WT plants did (Fig. S4), confirming the previous conclusion that elevated flavonoid accumulation is not the primary reason for the growth defect in HCT-RNAi plants (4).

SA-Mediated Growth Effects in Low-Lignin Plants May Operate via GA Signaling. HCT-RNAi alfalfa plants show reduced GA levels and perception (11). To test whether the dwarf phenotype of Arabidopsis HCT-RNAi lines with elevated levels of SA is mediated via altered GA sensing or signaling, we first examined the effects of exogenous addition of GA4 to Arabidopsis seedlings germinated on Petri plates for 10 d. The GA biosynthesis inhibitor paclobutrazol caused total inhibition of growth of both WT and HCT-RNAi lines (Fig. 4A). Both WT and HCT-RNAi seedlings showed similar growth enhancement at GA concentrations of $10^{-6} \text{M}$ and above (Fig. 4A). However, at 8 wk postgermination, GA did, however, increase rosette diameter and petiole length in lines in which the HCT-RNAi was
exogenous GA application (Fig. 4 C–E). However, the GA responsiveness of these genes (repression for GA20 and GA3 oxidases and induction for GASA1 and XTH genes) was restored in HCT-RNAi lines in the sid2-2 and NahG backgrounds after exogenous GA application (Fig. S5 A–C), with the notable exception of XTH9 in sid2 lines (Fig. S5C).

**Discussion**

Down-regulation of lignin pathway genes in transgenic plants often leads to reduced biomass associated with altered growth and vascular morphology. This phenomenon has been observed in plants with partial knockdown of enzyme activities through expression of antisense or RNAi constructs (5, 7) and is more apparent in genetic knockouts, which are often highly dwarfed (4, 27, 28). If these growth effects are primarily caused by altered vascular morphology and function, it will be difficult to reverse-engineer low-lignin plants for improved growth. This concept was questioned when it was proposed that metabolic spillover from the lignin to the flavonoid pathway was the cause of the reduced growth phenotype in HCT–down-regulated Arabidopsis plants via flavonoid-mediated inhibition of auxin transport (3). However, although down-regulation of HCT clearly does result in increased flavonoid levels (3, 11), reduced growth in HCT–down-regulated alfalfa plants is not associated with alterations in auxin transport (11); flavonoids are elevated in some, but not all, reduced lignin lines with altered growth (2). Furthermore, reexamination of the original experiments linking flavonoids to growth effects in Arabidopsis (4), as well as the present analyses of flavonoid levels in Arabidopsis plants with low lignin but restored growth, strongly support the argument that flavonoids are not causally linked to growth defects in lignin-modified plants but that SA levels are.

Two pathways have been postulated for SA biosynthesis in plants: from cinnamate via benzoate (19) or from shikimate via isochorismate (18). The fact that the elevated SA levels present in HCT-RNAi plants were reduced to WT levels in HCT-RNAi plants homozygous for the sid2 mutation suggests that lignin down-regulation induces SA formation primarily through the isochorismate pathway, which operates in Arabidopsis during responses to pathogens (18). The correlation between SA levels and the proportion of cold-water–extractable pectin to total pectin (Fig. 1B) in multiple transgenic reduced lignin alfalfa lines is consistent with the hypothesis that release of pectic elicitors from underlignified secondary cell walls in HCT-RNAi lines induces SA, and thereby defense responses, in the same manner as would occur when pathogens degrade cell walls during ingress (11, 12) rather than that the SA accumulates simply as a result of metabolic spillover from the monolignol pathway. A program of secondary wall formation with limited lignification can result in pectin that is more extractable (11), and pectic oligosaccharides are well known as elicitors of plant defense responses (12, 29, 30).

HCT-RNAi alfalfa (11) and Arabidopsis (the present work) are nonresponsive to GA as a growth enhancer when beyond the early seedling stage. GA-induced growth is restored upon reduction of the SA pool in Arabidopsis, suggesting that SA may mediate its growth-reducing effects through GA signaling. Our data do not support an alternative model in which reduced growth is largely a result of the diversion of energy into defense responses (e.g., PR protein expression). Although SA is increasingly being shown to impact plant growth (15), there has been little study of its potential involvement in elongation growth. Our work therefore provides a unique system for studying the poorly understood cross-talk between plant defense and growth control mechanisms (31, 32). In conclusion, we have shown that SA-mediated events are central to the orchestration of the reduced growth response of HCT-RNAi plants. This finding has clear implications for the engineering of improved bioenergy crops with optimal agronomic performance.
Materials and Methods

Plant Material. Arabidopsis HCT-RNAi lines were obtained from Purdue University and were as previously described (4). Plants used for crossing were the mutant alleles sid2-2 (18, 20) and npr1-1 (24) along with plants expressing the NahG transgene (33); all were in the Columbia-0 ecotype background. The HCT-RNAi transgene was detected by RT-PCR using the primer pair reported previously (4). Mutants carrying the npr1-1 mutation were identified with the codominant amplified polymorphic sequences (CAPS) markers described in ref. 24. Arabidopsis ccr1 (AT1G15950.1) mutant seeds were obtained from the Biological Resource Center (line SALK_123689). Primer pairs for plant genotyping were as follows: left primer, 5′-TGTGGTAGGCGGCTTG-3′; and LBb1.3, 5′-ATTGCGGACCCGGAC-3′. Alfalfa antisense lines analyzed were described previously (5, 34, 35). For the specific lines used, see SI Materials and Methods.

GA Application. Treatments with GA4 were as described in SI Materials and Methods.

Plant Growth Measurement and Histochemical Analysis. Plant height was measured as described in SI Materials and Methods. Maüle staining for lignin was performed as previously described (7) in cross-sections from the base of the mature stems.

Determination of SA Levels. SA levels were determined by using the biosensor organism Acinetobacter sp. ADPWH_Lux as described previously (36, 37) and in SI Materials and Methods.

Assay of HCT Activity and Determination of Lignin and Flavonoid Levels. HCT activity was determined as described in SI Materials and Methods. Lignin content and composition was determined by thiocacidolysis (38). Soluble phenolic compounds were extracted by using the protocol described in ref. 4 and as further described in SI Materials and Methods.

Determination of Pectic Compounds. Extraction of pectic materials from cell-wall residues was determined as described previously (11). The proportion of pectic material released by cold-water extraction was determined as a proportion of total pectin.

Measurement of Transcript Levels by qRT-PCR. qRT-PCR analysis was performed as described previously (11). Gene-specific primers are listed in Table S2.

Statistical Analysis. Statistical treatment of data was performed by ANOVA using Fisher's least significant difference procedure for multiple-comparison tests (Statgraphics Plus, version 5.1 for Windows). Significance of correlations was obtained by using the online calculator for Pearson correlation P values at http://www.danielsoper.com/statcalc/calc44.aspx.

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**Supporting Information**

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**SI Materials and Methods**

**Plant Material and Growth Conditions.** Arabidopsis hydroxycinnamoyl CoA: shikimate hydroxycinnamoyl transferase (HCT)-RNAI lines were obtained from Purdue University and were previously described (1). Plants for crossing were the mutant alleles sid2-2 (2, 3) and npr1-1 (4) along with plants expressing the NahG transgene (5); all were in the Arabidopsis thaliana Columbia-0 ecotype background. Seeds were exposed to a temperature of 4 °C for 2 d and then sown in a seedling mix substrate with controlled fertilizer at 21 °C under a 16-h light/8-h dark photoperiod. Light intensity was 110 µmol·m−2·s−1, supplied by both incandescent and fluorescence lights. The HCT-RNAI transgene was detected by RT-PCR using the primer pair reported previously (1). Primer pairs for detecting the sid2-2 mutation and the NahG insertion were as follows: sid2-2, 5′-ATTGGGAGGGAGCTAAGGAA-3′ and 5′-TCTTCTATCGAATGTATTCTATTCCTT-3′; and NahG, 5′-CAGGCGGGATGATTCAT-3′ and 5′-CAGGAGATTTGGGGATTACCC-3′. Mutants carrying the npr1-1 mutation were identified with the codominant amplified polymorphic sequences (CAPS) markers described in ref. 4. Plants were genotyped before biochemical or gene-expression analysis. Arabidopsis cinnamoyl CoA reductase 1 (ccr1) (AT1G15950.1) mutant seeds were obtained from the Arabidopsis Biological Resource Center (line SALK_123689). Primer pairs for plant genotyping were as follows: left primer, 5′-GTGTCGTAGAGGCTTTGCTTG-3′; right primer, 5′-TTTGAGAATTATTTGGGTGT-3′; and Lbb1.3, 5′-ATTGGGAGGGAGCTAAGGAA-3′. Alfalfa antisense lines were as described previously (6–8). The specific lines used were 4CL (34a, 13a, and 25a), C3H (4a, 9a, and 5A), HCT (30a, 3a, and 29a), CCaOMT (CC2-305), CCR (48a, 4a, and 41a), CAD (17a, 63a, and 56a), COMT (C2-310), an empty control vector (CK48), and two WT lines (Ctrl1 and Ctrl49).

**Gibberellin (GA) Application.** Arabidopsis seeds were grown on Murashige and Skoog medium supplemented with 10−2, 10−6, and 10−5 M GA4 (Sigma-Aldrich), Paclobutrazol (10−8 M) and Murashige and Skoog medium alone were used as control treatments. Plates were placed in a growth chamber, plant growth was measured, and pictures were taken after 12 d of growth. For plants grown in soil, applications of 10−6 M GA4 were performed by spraying plants and watering soil twice per week for 2 wk. Eight plants were used as biological replicates per treatment. Experiments were repeated three times.

**Plant Growth Measurement and Histochemical Analysis.** Plant height was measured from the base of the rosette to the tip of the primary inflorescence stem. Both plant height and rosette diameter were measured at mature stage when the primary stem had ceased elongation. Maule staining for lignin was performed as previously described (9) in cross-sections from the base of the mature stems.

**Determination of Salicylic Acid (SA) Levels.** SA levels were determined by using the biosensor organism Acinetobacter sp. ADPWM_Lux as described previously (10, 11). Stems (100 mg fresh weight) were ground in fresh LB liquid medium (2.5 mL of LB per 1 g of stem) by vortexing for 30 s and sonicking for 5 min on ice, and the homogenates were then centrifuged at 12,000 × g for 15 min. The supernatants were used for SA measurement, and an equivalent volume of LB medium was used to make an SA standard curve (SA final concentrations of 0, 0.05, 0.25, 0.5, 1.6, 8.3, 20, 40, 83, 166, and 200 µM). An overnight culture of Acinetobacter sp. ADPWM_Lux was diluted in LB medium (1:20) and grown at 37 °C for ~2 h to an OD600 of 0.4. Then, 60 µL of LB medium, 50 µL of SA biosensor culture, and 20 µL of each crude extract were mixed in a 96-well cell-culture plate. The plate was incubated at 37 °C for 1 h without shaking, and bio- luminescence and OD600 of negative controls (LB alone or water) were read with a GloMax-Multi Detection System (Promega). SA standards and negative controls were read in parallel with the experimental samples, and every sample was replicated five times. Relative bioluminescence was obtained by subtracting bio- luminescence OD600 of negative controls, and SA concentration was estimated according to the SA standard curve.

**Assay of HCT Activity.** Approximately 0.2 g of ground stem tissue was resuspended in 2.7 mL of extraction buffer [100 mM Tris Cl (pH 7.5), 10% glycerol, 1 mM PMSF, and 0.5 mM DTT] and 0.1 g of polyvinylpolypyrolidone was then added. The suspension was kept on ice for 45 min with occasional vortexing. The supernatant was recovered after centrifugation (12,000 × g for 5 min) and desalted by passing through a PD-10 column (GE Healthcare) according to the manufacturer’s instructions but with collection of only 1.5 mL of sample after the first 1.5 mL had been discarded. The protein concentrations of plant extracts were determined with the Bio-Rad protein assay. For enzyme assay, 3–5 µg of protein extract was incubated at 30 °C for 20 min with 100 mM sodium phosphate buffer (pH 7.5), 5 mM shikimic acid, 1 mM DTT (Roche), and 60 µM 4-coumaroyl CoA in a final volume of 100 µL. The reactions were stopped by adding 10 µL of glacial acetic acid, and products were analyzed by reverse-phase HPLC on a C18 column (Spherisorb 5 µ ODS2; Waters) in a step gradient using 1% phosphoric acid in water as solvent A and acetonitrile as solvent B. Chlorogenic acid (caffeylic quinic acid) was used to construct the calibration curve.

**Determination of Lignin and Flavonoid Levels.** Lignin content and composition was determined by thiaoacidolysis as described previously (12). Soluble phenolic compounds were extracted by using the protocol described in ref. 1. Forty microliters of the supernatant was injected onto a ODS2 reverse-phase column (5-µm particle size, 4.6 × 250 mm) and eluted in 1% (vol/vol) phosphoric acid with an increasing gradient of acetonitrile (0–5 min, 5%); 5–10 min, 5–10%; 10–25 min, 10–17%; 25–30 min, 17–23%; 30–65 min, 23–50%; 65–69 min, 50–100%) at a flow rate of 1 mL·min−1 for 70 min. The main peaks present in all samples were kaempferol-3-O-[rhamnosyl(1→2)glucoside]-7-O-rhamnosi- side (K1), kaempferol-3-O-glucoside-7-O-rhamnoside (K2), and kaempferol-3-O-rhamnoside-7-O-rhamnoside (K3). Compound identification was confirmed by liquid chromatography/MS. Flavanoid content was quantified by adding the K1, K2, and K3 peak areas at 320 nm (mAU) and using a kaempferol calibration curve as standard.

**Determination of Pectic Compounds.** Extraction of pectic materials from alcohol-insoluble cell-wall residues was determined as described previously (13). The proportion of pectic material released by cold-water extraction was determined as a proportion of total pectin.

**Measurement of Transcript Levels by Quantitative RT-PCR (qRT-PCR).** qRT-PCR analysis was performed as described previously (13). Gene-specific primers are listed in Table S2.
Statistical Analysis. Statistical treatment of data was performed by ANOVA using Fisher’s least significant difference procedure for multiple-comparison tests (Statgraphics Plus, version 5.1 for Windows). Significance of correlations was obtained by using the online calculator for Pearson correlation *P* values at http://www.danielsoper.com/statcalc/calc44.aspx.


Fig. S1. Silencing HCT induces PR gene expression and SA accumulation in *Arabidopsis*. (A) Expression level of HCT, PR5, PR1, and PR2 transcripts determined by qRTPCR in WT and two HCT-RNAi lines. The relative expression of mRNA was normalized to that of the *Arabidopsis* serine/threonine phosphatase (PP2A) and β-tubulin (BT) genes. Results are the means ± SD of three biological replicates, and each PCR was run in triplicate. (B) SA levels in WT and two HCT-RNAi lines. Results are means of six biological replicates.
Fig. S3. Effects of expression of NahG in HCT-RNAi Arabidopsis. (A) Extractable HCT enzyme activity. (B) Lignin content and composition as determined by thioacidolysis. H, hydroxyphenyl unit; G, guaiacyl unit; S, syringyl unit. (C) Rosette diameters and plant heights. A pool of three plants with the same genotype was considered to be one biological replicate, and results are the means ± SD of three biological replicates in the case of WT, NahG, and HCT-RNAi lines. Ten progeny plants from HCT-RNAi in the NahG background were analyzed independently for each parameter.

Fig. S2. Loss-of-function of the Arabidopsis CCR1 gene of monolignol biosynthesis induces SA accumulation and PR gene expression. (A) Expression level of PR1, PR5 and PR2 transcripts determined by qRT-PCR in WT and ccr1 mutant. The relative expression of mRNA was normalized to that of the Arabidopsis serine/threonine phosphatase (PP2A) and β-tubulin (BT) genes. Results are the means ± SD of three biological replicates, and each PCR was run in triplicate. (B) SA levels in WT and ccr1 mutant. Results are means of six biological replicates.
Fig. S4. Abolition of SA accumulation partially restores flavonoid accumulation to HCT-RNAi Arabidopsis. Flavonoid levels were determined by HPLC of extracts from stems of WT, sid2-2 null mutant, HCT-RNAi, and HCT-RNAi heterozygous or homozygous for the sid2-2 mutation. Values with different letters are significantly different (P < 0.05). A pool of three plants with the same genotype was considered to be one biological replicate, and results are the means ± SD of three biological replicates.

Fig. S5. Transcript levels of GA biosynthesis and response genes in Arabidopsis plants with and without GA application (8 d postapplication). Plants are sid2-2 null mutant, HCT-RNAi with homozygous sid2-2, NahG, and HCT-RNAi in the NahG background. Transcripts levels were determined by qRT-PCR, and values (means and SDs from three biological replicates) are given relative to the Arabidopsis serine/threonine phosphatase (AtPP2A) transcript levels. (A) GA20 and GA3 oxidase. (B) GASA1. (C) XTH4, XTH9, and XTH24. See Fig. 4 C–E for response of WT plants grown in parallel.
Table S1. Responsiveness of Arabidopsis lines HCT-RNAi, sid2-2, and NahG (alone and in the HCT-RNAi background), and control Arabidopsis lines to external application of GA₄

<table>
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<th>Line</th>
<th>GA₄</th>
<th>Rosette diameter (mm)</th>
<th>Inflorescence length (mm)</th>
<th>Petiole length (mm)</th>
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<tr>
<td>WT</td>
<td>−</td>
<td>53.5 ± 4.7*</td>
<td>148.2 ± 9.2*</td>
<td>10.0 ± 1.3*</td>
</tr>
<tr>
<td>WT +</td>
<td>+</td>
<td>76.5 ± 5.7†</td>
<td>198.2 ± 7.5†</td>
<td>14.0 ± 1.6†</td>
</tr>
<tr>
<td>sid2-2</td>
<td>−</td>
<td>42.5 ± 4.7*</td>
<td>139.7 ± 12.8*</td>
<td>9.3 ± 2.0*</td>
</tr>
<tr>
<td>sid2-2 +</td>
<td>+</td>
<td>68.5 ± 4.3†</td>
<td>179.7 ± 20.7†</td>
<td>12.4 ± 2.0*</td>
</tr>
<tr>
<td>HCT-RNAi</td>
<td>−</td>
<td>27.7 ± 8.0*</td>
<td>47.3 ± 5.9*</td>
<td>4.0 ± 1.4*</td>
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<tr>
<td>HCT-RNAi +</td>
<td>+</td>
<td>29.7 ± 7.1*</td>
<td>51.2 ± 5.5*</td>
<td>3.2 ± 1.1*</td>
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<tr>
<td>NahG</td>
<td>−</td>
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<td>142.5 ± 11.5*</td>
<td>8.7 ± 1.5*</td>
</tr>
<tr>
<td>NahG +</td>
<td>+</td>
<td>70.5 ± 5.1†</td>
<td>183.2 ± 19.3†</td>
<td>12.3 ± 2.0†</td>
</tr>
<tr>
<td>sid2-2/sid2-2 × HCT-RNAi</td>
<td>−</td>
<td>48.8 ± 6.1*</td>
<td>116.7 ± 19.2*</td>
<td>5.7 ± 1.2*</td>
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<tr>
<td>sid2-2/sid2-2 × HCT-RNAi +</td>
<td>+</td>
<td>58.5 ± 2.1†</td>
<td>130.0 ± 9.9*</td>
<td>7.8 ± 1.0†</td>
</tr>
<tr>
<td>NahG/NahG × HCT-RNAi</td>
<td>−</td>
<td>45.5 ± 3.9*</td>
<td>123.4 ± 10.3*</td>
<td>7.0 ± 1.0*</td>
</tr>
<tr>
<td>NahG/NahG × HCT-RNAi +</td>
<td>+</td>
<td>69.5 ± 2.3†</td>
<td>155.8 ± 16.7†</td>
<td>10.6 ± 2.0†</td>
</tr>
</tbody>
</table>

Rosette diameter, inflorescence length, and petiole length were measured at 15 d after application of 10⁻⁵ M GA₄ via spraying and watering soil. Plants were 8 wk postgermination at the start of the experiment. Results are means ± SDs of eight replicate plants per treatment. Values for individual genotypes with and without GA treatment are marked with different signs (* or †) if they are significantly different (P < 0.05). Statistical analysis was performed independently with each pool of data from each plant genotype.

Table S2. Primers used for qRT-PCR

<table>
<thead>
<tr>
<th>Gene-specific primer</th>
<th>Forward (5′ → 3′)</th>
<th>Reverse (5′ → 3′)</th>
</tr>
</thead>
<tbody>
<tr>
<td>AtPP2A (serine/threonine phosphatase)</td>
<td>AGATCGCTCGGAACCTGAA</td>
<td>ACATCCTCAGAAACTCTAA</td>
</tr>
<tr>
<td>AtBT (tubulin)</td>
<td>TGGGAACTGTGCTCATATCT</td>
<td>GAAAGGAATGAGGTACCTG</td>
</tr>
<tr>
<td>AtPR1</td>
<td>GTTCGCGCCGCGATGACGT</td>
<td>TGACGCTGGTTCGGGTACA</td>
</tr>
<tr>
<td>AtPR2</td>
<td>ACGCCTACCATCATCTAGACT</td>
<td>GAGTACCTGGATCGTAAACA</td>
</tr>
<tr>
<td>AtPR5</td>
<td>CGAAGGCGGCGAAAGATTT</td>
<td>CCCCAGGTACATGTTAAC</td>
</tr>
<tr>
<td>AtHCT</td>
<td>TCACCGGGGAGCTGTTTTT</td>
<td>CGAGCAATACAGACATG</td>
</tr>
<tr>
<td>AtGA20ox1</td>
<td>GATCCATCTCTTTCACATTA</td>
<td>GTGATATTGAGGCTCTG</td>
</tr>
<tr>
<td>AtGA3ox1</td>
<td>GAGACCTATCGTCTTTCCTTC</td>
<td>GAGGAGAAAGAGGGAC</td>
</tr>
<tr>
<td>AtGASA1</td>
<td>CTTTGATCAATTGGCTTCTTC</td>
<td>ACCATTTCTCTTCG</td>
</tr>
<tr>
<td>AtXTH24</td>
<td>TTCTCGTGAGGGGCCGTT</td>
<td>AGCTAGCTTTGCTGAC</td>
</tr>
<tr>
<td>AtXTH4</td>
<td>GTGCAGGACGAGGACCAC</td>
<td>TTGAGACGACGACACG</td>
</tr>
<tr>
<td>AtXTH9</td>
<td>CACTACCGCGAGCGCTTACA</td>
<td>TCTGGTTCAAACCTC</td>
</tr>
</tbody>
</table>